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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : C12N 5/00	A1	(11) International Publication Number: WO 91/18971 (43) International Publication Date: 12 December 1991 (12.12.91)
(21) International Application Number: PCT/US91/03269 (22) International Filing Date: 10 May 1991 (10.05.91) (30) Priority data: 529,623 29 May 1990 (29.05.90) US (71)(72) Applicants and Inventors: MOSCOVICI, Carlo [US/US]; MOSCOVICI, M., Giovannella [US/US]; 6816 Northwest 18th Avenue, Gainesville, FL 32605 (US). (74) Agent: CAHN, Maurice, U.; Leydig, Voit & Mayer, 700 Thirteenth Street, NW, Suite 300, Washington, DC 20005 (US).		(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent). Published <i>With international search report.</i>
(54) Title: NON-PRODUCER CELL LINES TRANSFORMED BY AMV (57) Abstract AMV-transformed non-producer cell lines which may be grown in serum-free media are disclosed. These cells have been transformed with the avian myeloblastosis virus oncogene, and are capable of being grown without chicken serum.		

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NON-PRODUCER CELL LINES TRANSFORMED BY AMVBackground of the Invention

Avian myeloblastosis virus (AMV) induces an acute myeloblastic leukemia in birds and is capable of transforming myeloid hematopoietic cells in vitro. AMV is a replication-defective virus and therefore requires a helper virus for replication.

Some of the features of this virus include: it is replication-defective; it is able to transform cells of hemopoietic origin (except fibroblast cells) together with cells of connective tissue origin; and finally it can induce leukemia in the chicken after short periods of latency.

Previous experiments have shown that after infection of hemopoietic cells with AMV, it is possible to obtain continuous lines of nonadherent transformed cells that can permanently release infectious virus. These cells are also found to carry distinctive markers of myeloid differentiations.

Summary of the Invention

Cell lines according to the present invention are non-producer avian cells transformed by avian myeloblastosis virus, and are capable of being grown in media which does not include chicken serum or phosphate. These cells are particularly suited for the production of avian vaccines.

Statement of Deposit

Cell lines according to the present invention

have been deposited in the American Type Culture Collection on May 25, 1990, thus affording permanency of the deposit and ready availability to the public upon issuance of a patent. Cell line

5 BM2L has received ATCC No. CRL 10468; cell line BM2L/A₁b₆ (also known as GM1243/1) has received ATCC No. CRL 10465; cell line BM2L/A₂a₂ (also known as GM1243/2) has received ATCC No. CRL 10466; and cell

10 line BM2L/A₂b₅ (also known as GM1243/3) has received ATCC No. CRL 10467.

Description of the Preferred Embodiments

The present invention comprises an immortalized or continuous cell line of non-producer chicken cells transformed by avian myeloblastosis virus

15 (AMV). In a preferred embodiment, a cell line of the invention grows in a culture media in the absence of tryptose phosphate broth and chicken serum.

Non-producer clones of myeloblasts transformed

20 by AMV are known [Moscovici, et al., In Vivo and In Vitro Erythropoiesis: The Friend System, G.B. Rossi, ed. (Amsterdam: Elsevier-North Holland), pp. 503-514 (1980)]. A non-producer cell line GM 727, a myeloblastic cell line originating from SPAFAS

25 embryonic bone marrow cells infected with AMV, were prepared in soft agar and developed into colonies. The GM 727 cells exhibit characteristics of monoblasts: they are positive for Fc receptors, can phagocytize latex particles, are negative for

30 complement receptors and immune phagocytosis, and produce colonies indistinguishable from AMV producer monoblasts in soft agar. Some virus-specific products are made by these cells, and infectious virions were never detected by the reverse

transcriptase assay or conventional biological assays.

Supernatant fluids from actively growing colonies of GM 727 were tested for production of virus by reverse transcriptase and conventional biological assays, such as transforming assay and the plaque assay. The BM2 cell line originated from the recovery of cells from the bone marrow of a bird injected in ovum with GM 727.

Typically, transformation of cells such as monoblasts, using viral agents such as AMV, is accomplished by flushing bone marrow out of chicken tibias, dispersing the cells in medium by vigorous pipetting, passing the cells through a fine mesh nylon cloth in order to obtain a single cell suspension, and then infecting the single cell suspension with the viral transformant. Colonies of cells transformed by AMV are easily recognizable morphologically using phase contrast microscopy, or by reverse transcriptase and conventional biological assays, such as transforming assay and the plaque assay.

Thereafter, the transformed monoblasts are serially sub-cultured to select the stably transformed cells from abortive or transiently transformed cells. The stable cells may then used to establish a line for use in accordance with the present invention. Specific details of a preferred transformation procedure are set forth in the Examples below.

In accordance with the present invention, bone marrow cells, preferably from a chicken embryo, are infected with avian myeloblastosis virus, resulting in the production of a transformed cell line. Infection typically occurs by culturing the bone

marrow cells with AMV. Included within the scope of the invention is culturing hematopoietic chicken cells with AMV, or a portion thereof, such as the v-myb or c-myb genes. Duesberg, et al., PNAS, 5 77:5120-5124 (1980) and Gonda, et al., Cell, 23:279-290 (1981) [both herein incorporated by reference] disclose the genetic structure of AMV. The AMV oncogene, v-myb, and a plasmid which may be used for the expression of the oncogene, are disclosed in 10 Klemmner, et al., Cell, 33:345-355 (1983), and is also herein incorporated by reference. Preferred transformed cells are disclosed in the Moscovici, et al. publication cited above.

In a preferred embodiment, BM2 cells, an AMV- 15 transformed non-producer cell line [described in Moscovici, et al., Expression of Differentiated Functions in Cancer Cells, R.F. Revoltella et al., eds. (New York: Raven Press) pp. 435-449 (1982), herein incorporated by reference] were used to 20 produce cells capable of being cultured in chicken serum-free media. The GM 727 cell line was produced from GM 727 cells, as described in Example 1. The BM2L cell line was produced according to Example 2, and was used to produce GM 1243/1, GM1243/2, and GM 25 1243/3, as described in Example 3.

Incubation of the parental cells according to the present invention is typically conducted in the presence of a nutrient medium which maintains the cells at temperatures permitting propagation of the 30 virus in the cell culture. Typically, such temperatures are from about 33°C to about 39°C, preferably about 37°C. The nutrient medium may be, for example, Eagle's Minimum Essential Medium (EMEM), Williams Medium E, Medium 199, Dulbecco's 35 Modified Eagle's Medium, Roswell Park Memorial

Institute culture media (RPMI-1640) or Basal Medium Eagle with, for BM2 and BM2L cells, chicken serum, tryptose phosphate broth, and calf (preferably newborn) serum. Other equivalent media may be used as well. The medium may contain a sufficient quantity of a buffering agent, such as sodium hydrogencarbonate, to maintain a stable pH. Other ingredients such as folic acid may be added to the culture medium in order to promote cell growth, particularly cell growth in suspension. Antibiotics, such as gentamycin, penicillin, streptomycin, and the like are advantageously included in order to prevent bacterial and/or fungal contamination of the culture. Typical media are shown in Example 4.

In a preferred embodiment, the cell lines according to the present invention -- BM2L/A₁b₆, BM2L/A₂a₂, and BM2L/A₂b₅, and their progeny, clones, variants, and mutations -- are grown in a medium such as one of those noted above, but which does not contain chicken serum or a phosphate such as tryptose phosphate. Such serum-free cells may be typically maintained in serum-free medium for about 48 hours. In a preferred embodiment, if long term maintenance is desired, a non-chicken serum, such as newborn calf serum, may be added.

The invention extends to the use of the serum-independent avian cell lines according to the present invention for the preparation of biologically active compounds or vaccines under serum-free conditions.

The terms "immortalized" or "continuous" as used herein means that the cell line grows continually without senescence when cultured in vitro in a suitable growth medium.

"Non-producer" as used herein refers to cells which do not release infectious virus. Typically, non-producer cells contain AMV viral sequences but do not contain the genome of the helper virus.

5 The term "serum-free medium" as used herein refers to a medium which is deprived of chicken serum, and/or a phosphate, such as tryptose phosphate. Chicken vaccines produced using the serum-free cell lines of the present invention do
10 not include the contaminants normally associated with chicken serum.

 A transformed cell line, i.e., cells which have been infected by AMV, may be detected in vivo by growing the cells infected with the virus in one of
15 several selective media. The labeling procedure typically includes culturing non-producer cells in a selective medium which contains ^{125}I -iodo-2-deoxyuridine ($^{125}\text{IUDR}$), a labelled nucleoside which can be radioactively measured, and Tris buffer.
20 SPAFAS embryonic bone marrow cells transformed with AMV are cultured in a $^{125}\text{IUDR}$ medium and the percentage of labelled cells can be determined by autoradiography. Labelled cells are those containing whole provirus DNA in the living cell.
25 Other methodologies for detecting the transformed cells in vivo include tagging the cells with beta-galactosidase.

 Advantages of the cell lines include an enormous reduction in costs since serum is very
30 expensive; cheap and simple purification of products produced by the new cell lines because there are no serum contaminants and because product can be easily purified from serum-free medium; the possibility of producing proteins which, due to the presence of
35 proteases in serum, cannot be obtained, or can only

be obtained in small quantities, from serum-dependent cell lines.

Utility of Cell Lines

- 1) Production of chicken-serum free vaccines:
5 the production of chicken vaccines using a continuous cell line which grows without chicken serum is significantly easier and much less costly because all that is needed is a cell suspension and an infecting agent (a chicken cytopathic virus or
10 virus, for example). Advantages include large volume production of vaccine at a relatively small cost. Some of these vaccines include, but are not limited to, Newcastle's disease, Fowl Pox, avian encephalomyelitis and avian infectious bronchitis.
- 15 2) Identification of potential therapeutic drugs or vaccines: These cells are useful for screening drugs or vaccines for the treatment of leukemia and related diseases, by growing the cells in media which contains the vaccine and then
20 determining extent cytotoxicity occurs, e.g., trypan blue exclusion assay or related assays, or by growth assays such as colony forming efficiency, all of which are standard techniques well known in the art.
- 3) Studies of chromosome damaging agents:
25 substances known or suspected of causing chromosomal damage may be added to a culture medium containing these cells, and then the extent of chromosomal damage can be measured.
- 4) Studies of malignant transformation by
30 viral agents and transferred genes, including oncogenes, using standard assays such as anchorage independent growth or tumor formation.
- 5) Use of cells altered by transfer of oncogenes of paragraph 3 above to screen for

potential therapeutic agents by the techniques noted in paragraph 1 above, particularly for cells transformed by the activation of certain oncogenes or combination of oncogenes.

5 6) Studies of cellular biochemistry, particularly those related to cell growth and the action of exogenous agents (such as those described in paragraphs 1-4 above).

10 7) Studies of cellular responses to growth factors and production of growth factors. These cells are particularly useful for such applications because they grow in defined growth media such as chicken-serum free and phosphate free media.

15 8) A model for the study of metastasis: the biological regulation of metastasis has not yet been determined or elucidated. The metastasis puzzle is what triggers a non-metastatic cell to become metastatic. By comparing the BM2 cell line (which remains in the bone marrow in vivo) to the BM2L cell
20 line (which migrates in vivo through the blood stream to other organs and tissues), information about the cellular or molecular changes in a metastatic cell may be determined.

25 While the invention is susceptible to various modifications and alternative forms, certain specific embodiments thereof are described in the examples set forth below. It should be understood, however, that these examples are not intended to limit the invention to the particular embodiments
30 disclosed, but, on the contrary, the intention is to cover all modifications, equivalents, and alternatives falling within the spirit and scope of the invention.

35 In order that the invention herein described may be more fully understood, the following examples

are for illustrative purposes only and are not to be construed as limiting this invention in any manner.

Examples

5 EXAMPLE 1. Development of the parental GM 727 cell line.

Bone marrow cultures were prepared from birds (baby chick SPAFAS) injected with NPs. The tibias were freed of all muscle and membranes and rinsed three times with Tris buffer. The cells were
10 obtained by flushing media through the bone marrow with a syringe. The cell clumps were broken up by pipetting and were passed through a nylon cloth in order to produce a single cell suspension. 1×10^6 of the cell suspension was then resuspended in 0.5
15 ml serial dilutions of biologically cloned AMV (MAV-2). Viral adsorption was carried out for 30 minutes at +4°C followed by 30 minutes at room temperature. Infected cells were then seeded at 5×10^4 per 35 mm dish, incubated at 37.5°C in 5% CO₂ and colonies were
20 scored and picked 10 days later.

Individual colonies were picked and seeded into multi-well plates and propagated in growth medium. The NP AMV myeloblasts were grown at a density of $6-10 \times 10^6$ cells/ml in BT-88(4) medium plus 160 µg/100
25 ml of folic acid. Supernatant fluids from individual colonies were harvested, filtered and tested for presence or absence of virus release by reverse transcriptase assay and by transformation assays of susceptible target cells.

30 Transformed non-producer monoblast cells were obtained from solitary infection of cells with the 10^{-3} virus stock dilution.

One non-producer clonal population was selected and labelled GM727.

1 x 10⁶ GM727 cells suspended in 100 µl medium were injected into the chorion allantoic membrane vein of 12 day old chicken embryos.

After hatching, chickens were monitored for onset of leukemia by blood smears twice a week. All experimental chicken remained leukemia negative. Once a week individual birds were sacrificed and their respective bone marrows were cultured in vitro. Monoblastic transformed cells were consistently reisolated from 1 week, 2 week, 3 week, and 4 week birds. From a 2 week old chick bone marrow, transformed cells were reisolated and a continuous line was obtained and named BM2. Cells from BM2 were cloned and one clone was selected and labelled BM2 C₃A.

The continuous BM2 C₃A cell line and its progeny has maintained its phenotype, biological properties, and lack of leukemogenicity for many years making it a unique chicken line.

20 EXAMPLE 2. Development of the BM2L cell line.

A chicken injected with BM2/C₃A showed anemia accompanied by runting. The bird was immediately sacrificed and autopsied.

Macroscopical and microscopical examination revealed microtumors in spleen, liver, heart, and bone marrow. The hystopath confirmed a leukemic process.

Leukemic bone marrow cells were cultured in vitro. The cells grew very actively with a generation time of 48 hours.

The cells were subcloned, and two clonal populations selected, and labelled BM2/LA₁, and BM2/LA₂, respectively. Supernatant fluids from the above cultures were collected and tested by reverse

transcriptase assay, which indicated that they were still non-producer.

DNA analysis by Southern Blot of BM2 C₃A, BM2/LA₁ and BM2/LA₂ cells showed no detectable change
5 in the "v-myb" gene of AMV.

Moreover, BM2/LA₁ when reinjected in chicken embryo again caused acute leukemia.

EXAMPLE 3.

Clonal populations of BM2/L can be grown in the
10 absence of tryptose phosphate broth and chicken serum. Serial dilutions of cells BM2/LA₁ and BM2/LA₂ were seeded in soft agar medium composed as follows:

Agar Medium

15	F-12 (2x)	20%
	F-12 (1x)	10%
	Fetal bovine serum	10%
	Dulbecco modified medium 1x	40%
	Vitamin 100x	1%
20	Folic Acid 100x	1%
	Gentamycin	

Growth Medium

25	Dulbecco modified medium 1x	88%
	Newborn calf serum	10%
	Vitamin 100x	1%
	Folic acid 100x	1%
	Gentamycin	
	pH	7.2

Colonies were picked 10 days later from
cultures seeded at 1×10^3 cells/35mm dish and
30 propagated in growth medium. Cell progenies from three colonies were selected, i.e., GM 1243/1, GM1243/2, and GM 1243/3.

EXAMPLE 4. Medium For Parental.

<u>F12 OVERLAY</u>	<u>20ml</u>	<u>30ml</u>	<u>40ml</u>	<u>50ml</u>	<u>60ml</u>	<u>70ml</u>	<u>80ml</u>	<u>90ml</u>	<u>100ml</u>
F12 (2X)	4.0	6.0	8.0	10.0	12.0	14.0	16.0	18.0	20.0
Calf Serum	1.2	1.8	2.4	3.0	3.6	4.2	4.8	5.4	6.0
Chickén Serum	0.4	0.6	0.8	1.0	1.2	1.4	1.6	1.8	2.0
Tryptose									
Phosphate	2.0	3.0	4.0	5.0	6.0	7.0	8.0	9.0	10.0
100X Vitamins	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
100X Folic Acid	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
Conditioned Medium*	8.0	12.0	16.0	20.0	24.0	28.0	32.0	36.0	40.0
Gentamycin									
Bacto Agar	4.0	6.0	8.0	10.0	12.0	14.0	16.0	18.0	20.0
(3.6% or (1.8%))									

*Conditioned Medium: Consisting of supernatant fluids of primary chicken embryo cells (from 9 day old embryo) after 4 days in culture.

Dulbecco's Modified Eagle MediumBase Medium

1-liter package of Dulbecco's Modified Eagle Medium powder obtained from GIBCO --- Formula #78-5 5440, to which may be added 2.2g of NaHCO_3 and 1 liter of water, filter sterilize and aliquot into sterile bottles.

Complete Medium

	Dulbecco's Modified Eagle Medium	80 ml
10	Tryptose Phosphate Broth (29.5g/l)	10 ml
	Calf Serum	5 ml
	Chicken Serum (heat inactivated)	5 ml
	Gentamicin (10 mg/ml)	0.1 ml

15 Although the foregoing invention has been described in some detail by way of illustration and example, it should be understood that the invention is not limited thereto, and that many obvious modifications and variations thereof can be made,
20 and that such modifications are intended to fall within the scope of the appended claims.

What is claimed is:

1. An continuous cell line comprising chicken cells transformed by nucleotide sequences from an avian myeloblastosis virus, said transformed cells
5 being capable of being cultured in a culture media substantially free of phosphate and chicken serum.
2. The cell line of Claim 1 wherein said phosphate is tryptose phosphate.
3. The cell line of Claim 1 wherein said cell
10 line is a non-producer.
4. The cell line of Claim 1 wherein said nucleotide sequences substantially correspond to the myb gene of avian myeloblastosis virus.
5. A cell line comprising chicken cells which
15 are substantially similar to cells selected from the group consisting of ATCC No. CRL 10465, and progeny or mutations thereof, ATCC No. CRL 10466, and progeny or mutations thereof, and ATCC No. CRL 10467, and progeny or mutations thereof.
- 20 6. A composition comprising at least one cell line selected from the group consisting of ATCC No. CRL 10465, and progeny or mutations thereof, ATCC

No. CRL 10466, and progeny or mutations thereof, and
ATCC No. CRL 10467, and progeny or mutations
thereof; and a medium therefor.

7. The cell line of Claim 1 wherein said cell
5 line is substantially similar to the cell line
consisting of ATCC No. CRL 10465, and progeny or
mutations thereof.

8. The cell line of Claim 1 wherein said cell
line is substantially similar to the cell line
10 consisting of ATCC No. CRL 10466, and progeny or
mutations thereof.

9. The cell line of Claim 1 wherein said cell
line is substantially similar to the cell line
consisting of ATCC No. CRL 10467, and progeny or
15 mutations thereof.

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US91/03269

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all.) According to International Patent Classification (IPC) or to both National Classification and IPC IPC(5): C12N 5/00 U.S.Cl.: 435/240.2		
II. FIELDS SEARCHED Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
US	435/240.2	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
APS, CAS, BIOSIS		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
Y	Cell, Volume 33, issued June 1983, K.H. Klempnauer <u>et al.</u> , "The product of the Retroviral Transforming Gene <u>v-myb</u> is a truncated version of the protein encoded by the cellular oncogene <u>c-myb</u> ," pages 345-355, see entire document.	1-4
Y	Molecular and Cellular Biology, Volume 4, No. 12, issued December 1984, G. Symonds <u>et al.</u> , "Induced Differ- entiation of Avian myeloblastosis Virus- Transformed Myeloblasts: Phenotypic Alteration Without Altered Expression of the Viral Oncogene," pages 2587-2593, see entire document but particularly page 2587, Materials & Methods, first paragraph.	1
<p>¹⁰ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"Z" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search		Date of Mailing of the International Search Report
04 June 1991		02 JUL 1991
International Searching Authority		Signature of the International Searching Authority Suzanne Ziska
ISA/US		

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X Y	Cell, Volume 31, issued December 1982, K. Radke <u>et al.</u> , "Transformation of Both Erythroid and Myeloid Cells by E26, an Avian Leukemia Virus That Contains the <u>myb</u> Gene", pages 643-653, see entire document.	1, 2, 4-9 1-9
Y	M.G. Moscovici <u>et al.</u> , " <u>In vivo</u> and <u>in vitro</u> Erythropoiesis: the Friend System", published 1980 by Elsevier/North-Holland Biomedical Press, see pages 503-514, see entire article.	1